MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF A NOVEL SPECIES OF *UVARIA* (ANNONACEAE) WITH POTENTIAL MEDICINAL PROPERTIES

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ABSTRACT

The imperfectly known genus *Uvaria* (Annonaceae) comprises ca. 20 species of shrubs to small trees in the Philippines. During recent fieldwork at Valderrama, Antique, a suspected new species of *Uvaria* was collected. Based on morphology, this *Uvaria* species can be distinguished from other Philippine congeners by its large obovate-lanceolate leaves and pubescent carpels. Additional molecular characters inferred from two plastid DNA regions (*mat*K and *trn*L-F) were sequenced from this *Uvaria* species and *U. grandiflora* to gain more certitude on the identity of this *Uvaria* species. Phylogenetic analysis using Maximum Parsimony was carried out including 48 foreign *Uvaria* species and other Annonaceae genera derived from GenBank. Results showed that the divergent *Uvaria* species and *U. grandiflora* were nested among the SE Asian *Uvaria* subclade, with a strong support (BS = 91%). Therefore, we propose a new species, *Uvaria valderramensis* sp. nov. The first phytochemical assays on the leaf extracts of *U. valderramensis* showed the presence of triterpenes, sterols, tannins, flavonoids, phenols and alkaloids.

KEYWORDS: *Uvaria,* Annonaceae, *mat*K, *trn*L-F, phytochemical assays

INTRODUCTION

Uvaria L. (Annonaceae) is a species-rich genus of woody climbers and scandent shrubs with over 210 known species (Zhou *et al.,* 2010) widespread in wet tropical forests of Africa, Madagascar, continental Asia, Malesia, Northern Australia and Melanesia (Zhou *et al.,* 2012). The genus is distinct from the rest of the Annonaceae by having stellate hairs, valvate sepals, flowers with imbricate leathery petals, monadal inaperturate pollen and apocarpous, many-seeded fruits (Zhou *et al.,* 2009). Some *Uvaria* species have also been known to contain biologically useful metabolites and exhibit medicinal properties (*e.g.,* Tempesta *et al.*, 1982; Nkunya *et al.,* 1991; Lawrence *et al.,* 2003; Okwu & Iroabuchi, 2008; Wiart, 2006)

In the "Flora of Manila" (Merrill, 1915) and "Enumeration of the Philippine Flowering Plants" by Merrill (1923), the genus has been noted to be widely distributed with most of its species considered to be endemic. Among the listed distribution sites of the genus, the municipality of Valderrama in Antique province was said to have an indigenous species, *U. grandiflora.* Although Merrill's extensive work has greatly contributed in elucidating the Flora of the Philippines, most of the species of *Uvaria* are left untreated and remain to be discovered. Aside from *U. grandiflora,* another distinctive species of the genus was also collected in Valderrama.

Systematic studies, most especially in the Annonaceae, have already started employing molecular analysis utilizing various gene markers as source of numerous reliable characters (*e.g.,* Zhou *et al.,* 2009; 2010; Su & Saunders, 2009; Meade, 2005; Richardson *et al.,* 2004; Mols *et al.,* 2004). This has provided better resolutions for the phylogenetic delimitations of *Uvaria* but none of these studies included representatives of Philippine *Uvaria.* Therefore, this study is a first step towards the completion of a phylogenetic study including all Philippine *Uvaria* species. Using both *trn*L-F and *mat*K sequence data of the chloroplast DNA along with morphological analysis, the present study aimed to: (1) ascertain the identity and phylogenetic position of the distinctive *Uvaria* species and *U. grandiflora* from Valderrama; (2) provide a comprehensive morphological description of the suspected novel *Uvaria* species; and (3) determine phytochemical constituents of this *Uvaria* species.

MATERIALS AND METHODS

Taxon Sampling. Field collections of the *Uvaria* species were done in the municipality of Valderrama, Antique, in central Philippines (11°00'05.60"N; 122°07'45.58"E). Two isolates of the commonly known red-flowered *U. grandiflora* (VI014b, VI014r) and four isolates of the divergent yellow-flowered *Uvaria* species (VI011, VI012a, VI012b, VI017) were collected. Herbarium specimens of these plants were stored in the University of Santo Tomas Herbarium (USTH). Fresh leaf samples were dried in silica gel for DNA extraction (Chase & Hills, 1991). Reproductive parts were preserved in 70% ethanol (Radford *et al.*, 1974) for morphological details.

Morphological Analysis. General morphological features were recorded based on Simpson's (2006) character list, while specific features of *Uvaria* were based from published literature (*e.g.,* Merrill, 1919; Meade, 2005). Field observations such as habitat, habit, scent, and color were noted in the field. Recently collected specimens were compared with digital type specimens found in the database of foreign herbaria (*e.g.,* L, NY, US). Microscopic parts such as carpels and stamens were observed using a microscope fitted with a digital camera.

DNA Extraction. Total DNA was isolated from silica-gel dried leaves following the protocols of the DNeasy Plant Mini Kit®, Qiagen, Germany. Final elution volume was 120µL of purified DNA. Samples were stored in a -20^{o}C freezer.

Amplification and Sequencing. Two chloroplast gene regions (*mat*K and *trn*L-F) were selected in this study and compared with *Uvaria* sequences in the GenBank. For amplification and sequencing of the *mat*K region the primer pair, 390F and 1326R (Cuenoud *et al.,* 2002) was used, while universal primers c and f (Taberlet *et al.,* 1991) were used for the primer pair of the *trn*L-F region.

PCR cocktail was prepared with the following proportions: 15.85 µL of water, 2.5 µL of 10x buffer, 2.0 µL of $MgCl_2$, 1.5 µL of 2mM dNTP, 1.0 µL of 10 µM forward primer, 1.0 µL of 10 µM reverse primer, 0.15 µL of *Taq* DNA polymerase, and 1.0 µL of pure DNA template to make a total of 25 µL. PCR amplifications were performed in the Biometra T-Gradient Cycler® running at an initial melting phase of 3 min at 94^{o}C, followed by 30-35 cycles of 1 min at 93^{o}C, 1 min at 59^{o}C and 2 min at 72^{o}C. Final extension phase ran at 5 min at 72^{o}C.

After amplification, samples were then subjected for purification using the Qia-Quick PCR Purification Kit® from Qiagen, Germany. Total purified DNA yielded 70 µL which was sent to Macrogen, Seoul, Korea for sequencing.

Data Analysis. Retrieved sequences were manually edited using the CodonCode Aligner v.3.7.1. Edited sequences were then aligned with the acquired sequences from GenBank using the MacClade v.4.0 software.

Aligned sequences were then subjected to parsimony analysis using the PAUP* v.4.0b (Swofford, 2000). Phylogenetic trees were obtained using the following settings: heuristic searches, MULTREES option on, tree-bisections-reconnection (TBR) branch swapping, using 5000 random addition sequences. The consistency and retention index (Farris, 1989) was calculated to measure homoplasy. Statistical support was derived through Bootstrap (BS) analysis using the following settings: 10,000 replicates, MULTREES option off, nearest neighbour interchange (NNI) branch swapping, and five random addition sequences. Clades having a BS value above 90% were considered to be strongly supported.

Phytochemical Analysis. Crude extracts were obtained by grinding dried leaves using a Wiley mill. Powdered leaves were added in dichloromethane (DCM) and methanol (MeOH) (1:1) then drained after 3 d. Crude extracts were added with distilled water then petroleum ether (1:1). The water layer was extracted then added with DCM (1:1) further forming two layers. Water layer then was separated and extracted with butane. Layers with petroleum ether, DCM and butanol were developed into a 9:1 of ethanol-acetic acid and MeOH.

Developed thin layer chromatography (TLC) plates were separately prepared for Vannilin reagent (Petroleum ether layer), Dragendorff's reagent (Butanol layer), and potassium ferricyanide (DCM layer). The spotted TLC plates were allowed to stand with the solvent until the chromatograms of the extracts were obtained.

RESULTS AND DISCUSSION

Sequence variation of the two cpDNA markers. A total of 70 taxa was included for both *trn*L-F and *mat*K sequence analyses. Five new sequences from each gene markers are published here for the first time. The *trn*L-F region ranged from 560 base pair (bp) (*Goniothalamus elegans*) to 955 bp *(Melodorum fruticosum*) of the unaligned data set. *Uvaria grandiflora* (VI014) had a total of 891 bp, while the unknown *Uvaria* (VI012a, VI012b, VI017) yielded an average of 901 bp. As for *mat*K, the unaligned sequence ranged from 771 bp (*U. angolensis*) to 842 bp *(G. cheliensis*). All newly sequenced *Uvaria* species yielded a mean base content of 840 bp.

The aligned data matrices for the *trn*L-F and *mat*K yielded a total length of 1094 and 849 bp, respectively. Out of the length of the *trn*L-F, 164 (14.99%) were parsimoniously informative characters, compared to *mat*K which had 182 (21.43%). Despite the length of the *trn*L-F against *mat*K, the latter had provided more informative sites. In the heuristic search of both gene markers, *trn*L-F resulted to a total of 3050 parsimonious trees, while *mat*K produced a total of 1831 equally parsimonious trees (Table 1). Consistency and retention indices and tree length of the trees provided with both *trn*L-F and *mat*K are presented in Table 1.

Phylogenetic analysis. The combined data matrix had a total of 70 taxa included in the analysis. Out of the 1943 bp, 346 (17.81%) are parsimoniously informative characters. The heuristic search excluding the uninformative characters resulted to a total of 4881 equally parsimonious trees with a total length of 662 steps (Table 1). Consistency and retention indices were computed to be 0.678 and 0.859, respectively (Table 1.)

In the separate (not shown here but available from the corresponding author) and combined tree analyses (Fig.1), *Uvaria* is resolved to be monophyletic with strong support (BS = 91%). The collected *Uvaria* spp. from Valderrama, Antique were shown in the combined tree (Fig. 1) to be nested in the Southeast (SE) Asian *Uvaria* group. Sampled *Uvaria grandiflora* formed a subgroup together with *U. flava, U. grandiflora U. hirsuta and U. calamistrata* with a high support (BS = 95%).

The collected divergent yellow-flowered *Uvaria* formed a subgroup of its own with a strong support (BS = 99%) and it is not directly related to *U. grandiflora* (Fig. 1). The molecular data support the identity of yellow-flowered *Uvaria* as a true member of the genus but was not identical to *U. grandiflora.*

Morphology of the divergent **Uvaria.** Congruent to the molecular analysis the distinctive *Uvaria* species collected from Valderrama revealed morphological features distinct to the genus. Among these characteristics sensu Keβler (1993) and Zhou *et al.* (2009) are the following: stellate hairs, valvate sepals, partially or fully imbricate and subequal whorls of leathery petals.

Furthermore, the presence of solitary, leaf-opposed inflorescences (Fig. 2a), numerous stamens and pistils (Fig. 2b), and fused sepals (Fig. 2c) are features that are shared by other Philippine *Uvaria* species. Among the Philippine species, the divergent *Uvaria* highly resembles *U. panayensis* on the basis of leaf shape, texture and venation pattern, and fruits (Fig. 3). However, the divergent *Uvaria* species is different by having ovate to obtuse sepals (Fig. 2c) and densely pubescent carpels (Fig. 4) in contrast to the reniform sepals and glabrous carpels of *U. panayensis*. With these distinctions, we propose a new name for the divergent *Uvaria* species, *Uvaria valderramensis* Cabuang, Exconde & Alejandro.

TAXONOMIC TREATMENT

Uvaria valderramensis Cabuang, Exconde & Alejandro *sp. nov.* (Fig. 5)

Type: Philippines, Visayas, Province of Antique, Municipality of Valderrama, Barangay Bugnay (11^{o}00'05.60"N; 122^{o}07'45.58"E) 14 May, 2011, *Cabuang P.G., Exconde B. , Lemana B.O.C. VI011* (holotype, USTH!; isotypes, PNH! USTH!)

> Shrubs, ca. 3 to 7 m tall, branches cylindrical, greenish-brown with lenticels; young branches green. Leaves glabrous on the upper surface while sparsely pubescent along the midrib on the lower surface; subcoriaceous; blades ovate, 15-31 cm long, 6-13 cm wide; margin entire, apex acute, obtuse, or mostly acuminate; base slightly cordate; leaf venation brochidodromous with 15-18 (-21) secondary veins; petioles 6-11 mm.
>
> Inflorescence leaf-opposed, axillary, solitary or simple corymbs with 2 to 4 flowers per node. Flowers pedicellate, 1.0-3.5 cm long, 2.0-4.5 cm diameter. Sepals ovate to obtuse, united at the base. Outer petals ovate, 3 to 4 per flower, yellow, 1-2 cm long, 1.0-1.8 cm wide; inner petals 3-5 per flower somewhat smaller than the former. Stamens and carpels numerous, forming a round, globose structure. Anthers brown, basifixed, flattened, oblong to somewhat discoid in shape, and leathery. Carpel arranged in aggregates. Pseudostyle apical in position, cylindrical. Stigma dark brown, flattened, oblong, leathery, covered usually with nectar in their receptive pistillate phase. Fruit monocarps *ca.* 10-20+, torulose, with slight constrictions in between seeds, each segment obovoid in shape, yellow-green with a smooth, fleshy pericarp. Fruits 16-23 mm in length, 8.0-8.65 mm in diameter with 3-4 seeds.

Seeds globose to obovoid, flattened, 6.0-6.5 mm in diameter, exotesta reddish brown.

Notes. *Uvaria valderramensis* is closely allied to *U. panayensis* based on similarities in the leaf blade shape and corolla lobe shapes. The species is however different from *U. panayensis* by having a large ovate to obovate leaves, distinctly yellow corolla lobes, and densely pubescent carpels.

Phenology. Flowering May to June and fruiting from July to November

Distribution and Habitat. Endemic to the Municipality of Valderrama, Antique, Panay Island, Visayas, Philippines. In riverine forests, shaded areas, hanging on trees, in clay soils, 20-30 m away from Bugnay Elementary School.

Local name. Usog (Kinaray-a)

Etymology. The specific epithet is derived from the type locality.

Additional specimens examined. **Visayas:** Province of Antique, Municipality of Valderrama, 14 May, 2011. *Cabuang P.G., Exconde B., Lemana B.O.C. VI012, VI017.* (syntypes, PNH!; USTH! <2 sheets>)

***Phytochemical analysis of* U. valderramensis.** The phytochemical assay on the leaf extracts of *U. valderramensis* showed the presence of phenols, alkaloids, flavonoids, steroids, tannins and terpenes. (Table 2)

Several of these compounds found in the leaf extract had already been proven to yield various medicinal and biological properties. Triterpenes for instance were found to possess anti-cancer properties (Liby *et al.,* 2007). Phenols and flavonoids can confer anti-oxidant properties on plants, while the latter also provide a strong potential anti-microbial agent (Aminimoghadamfarouj *et al.,* 2011). Flavonoids have been proven to demonstrate anti-inflammatory properties (Okwu & Iroabuchi, 2008).

CONCLUSIONS

The present molecular and morphological study confirmed the placement of both *Uvaria grandiflora* and the unknown *Uvaria* collected in Valderrama, Antique to be members of the genus *Uvaria.* Maximum parsimony analyses and the morphological features of the unknown *Uvaria* species corroborated the distinction of this species from the rest of the SE Asian species. With this, we proposed a new species, *Uvaria valderramensis* Cabuang, Exconde & Alejandro *sp. nov.* This new species of *Uvaria* has yielded several phytochemical compounds.

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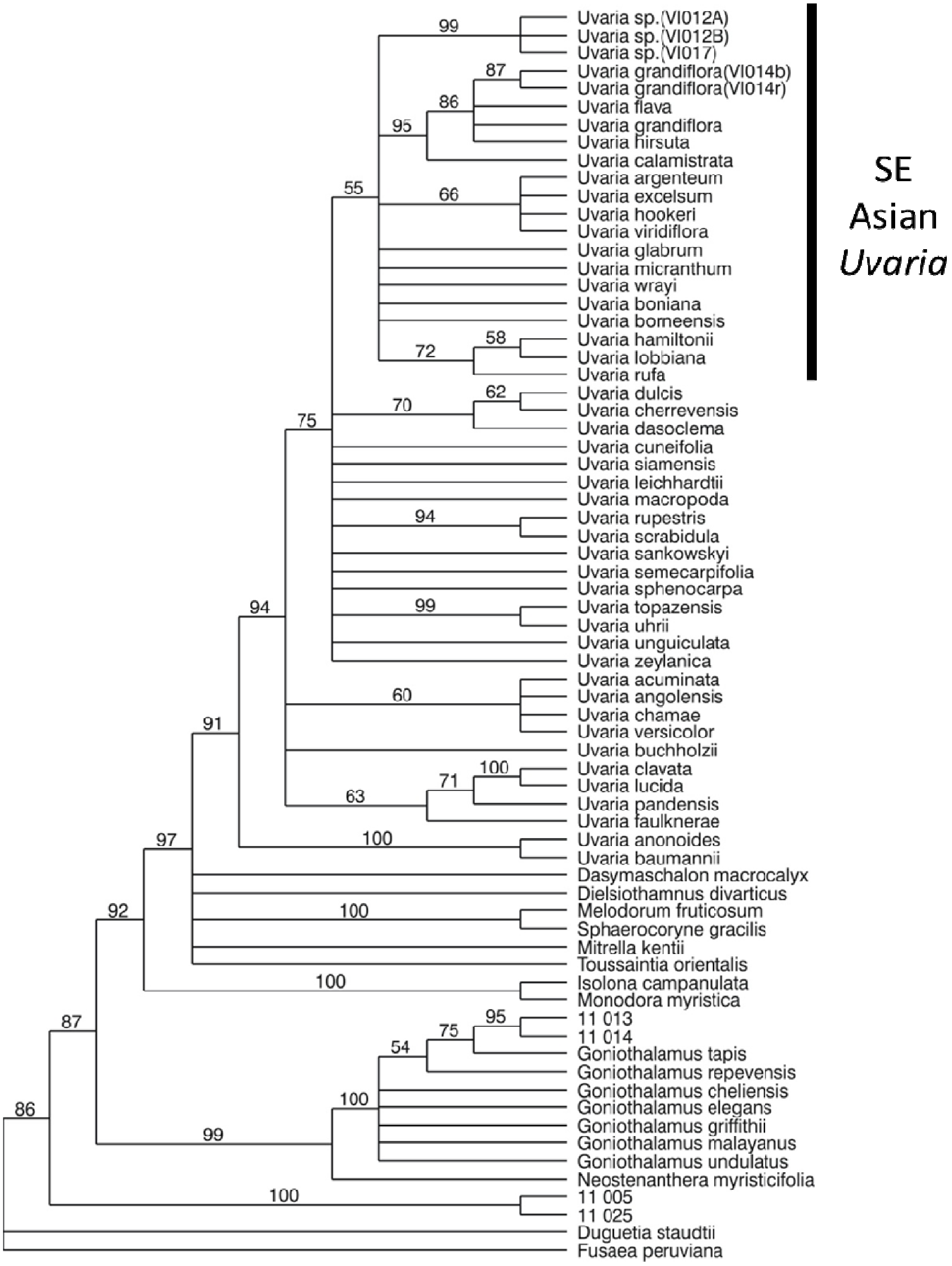

Figure 1. Strict consensus tree derived from 4881 equally parsimonious trees based on the combined analysis of *trn*L-F-*mat*K sequence data. Numbers above the nodes indicate bootstrap values >50%. *Uvaria* spp. included in the analysis bear their respective codes.

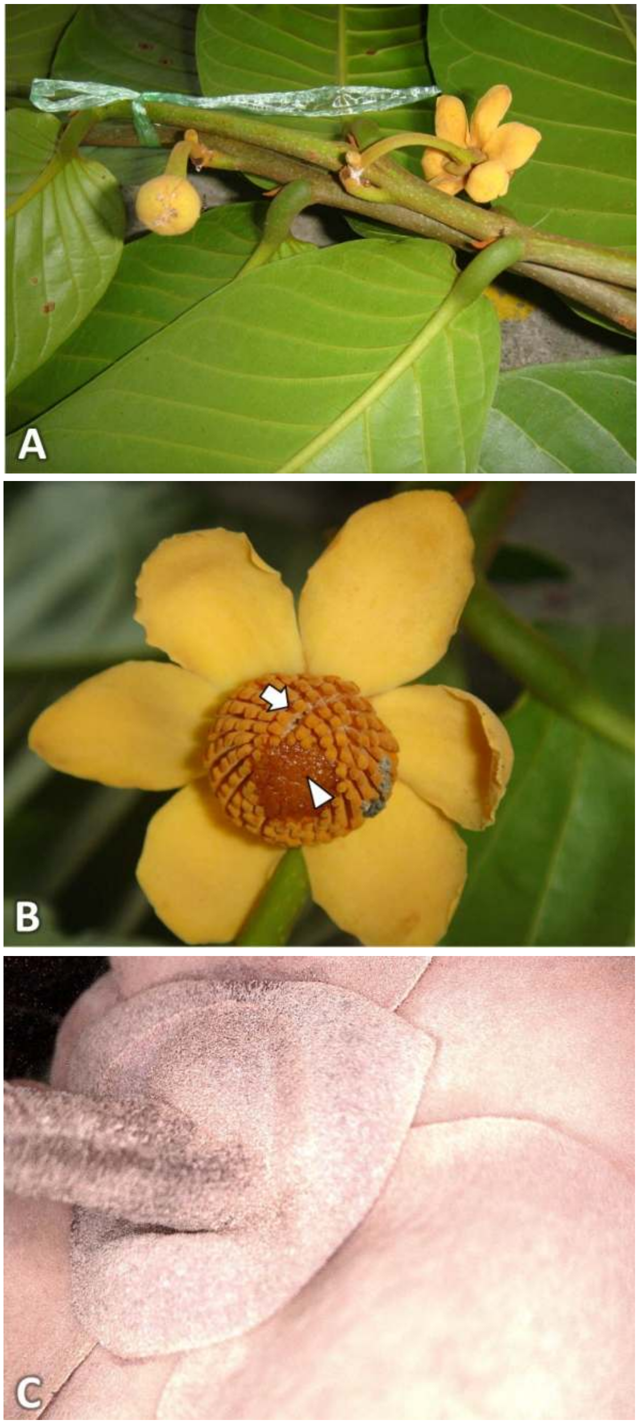

Figure 2. Uniting features of *Uvaria valderramensis* sp. nov. with the Philippine *Uvaria* group. A. Leaf-opposed inflorescence; B. Numerous stamens (arrow) and carpels showing the exposed stigma (arrowhead); C. Fused sepals.

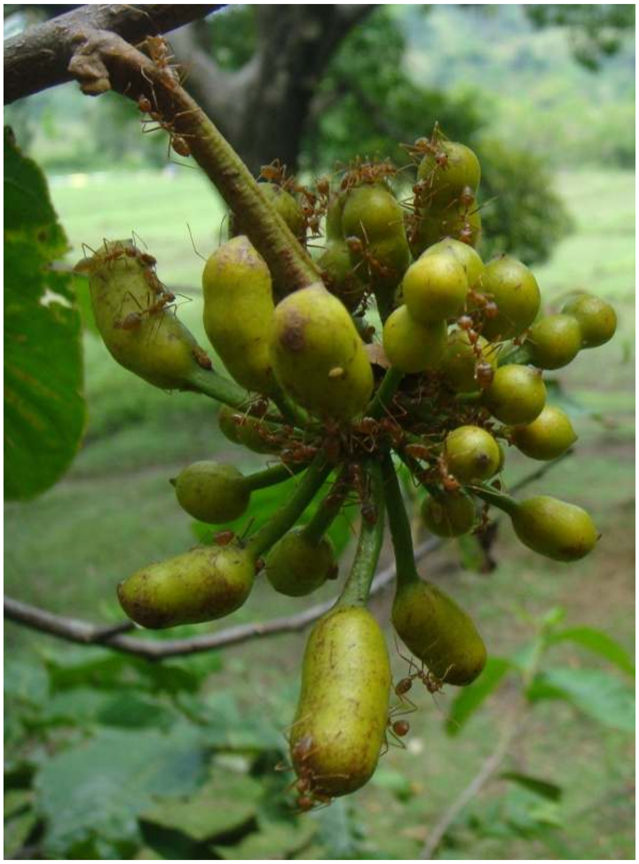

Figure 3. Fruits of the *Uvaria valderramensis* sp. nov.

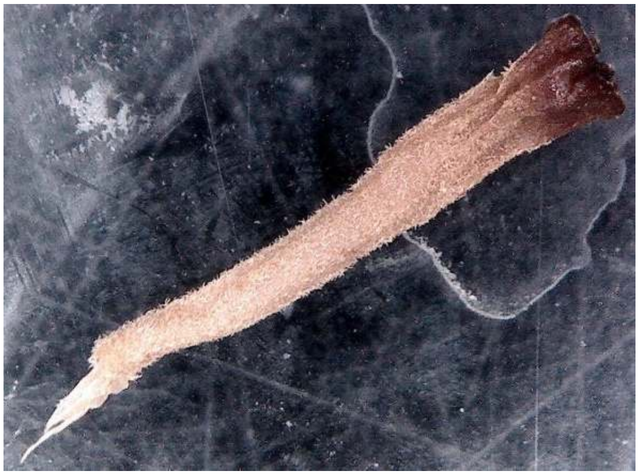

Figure 4. Magnified view of an individual carpel of the *Uvaria valderramensis* sp. nov. densely covered with trichomes. (10x)

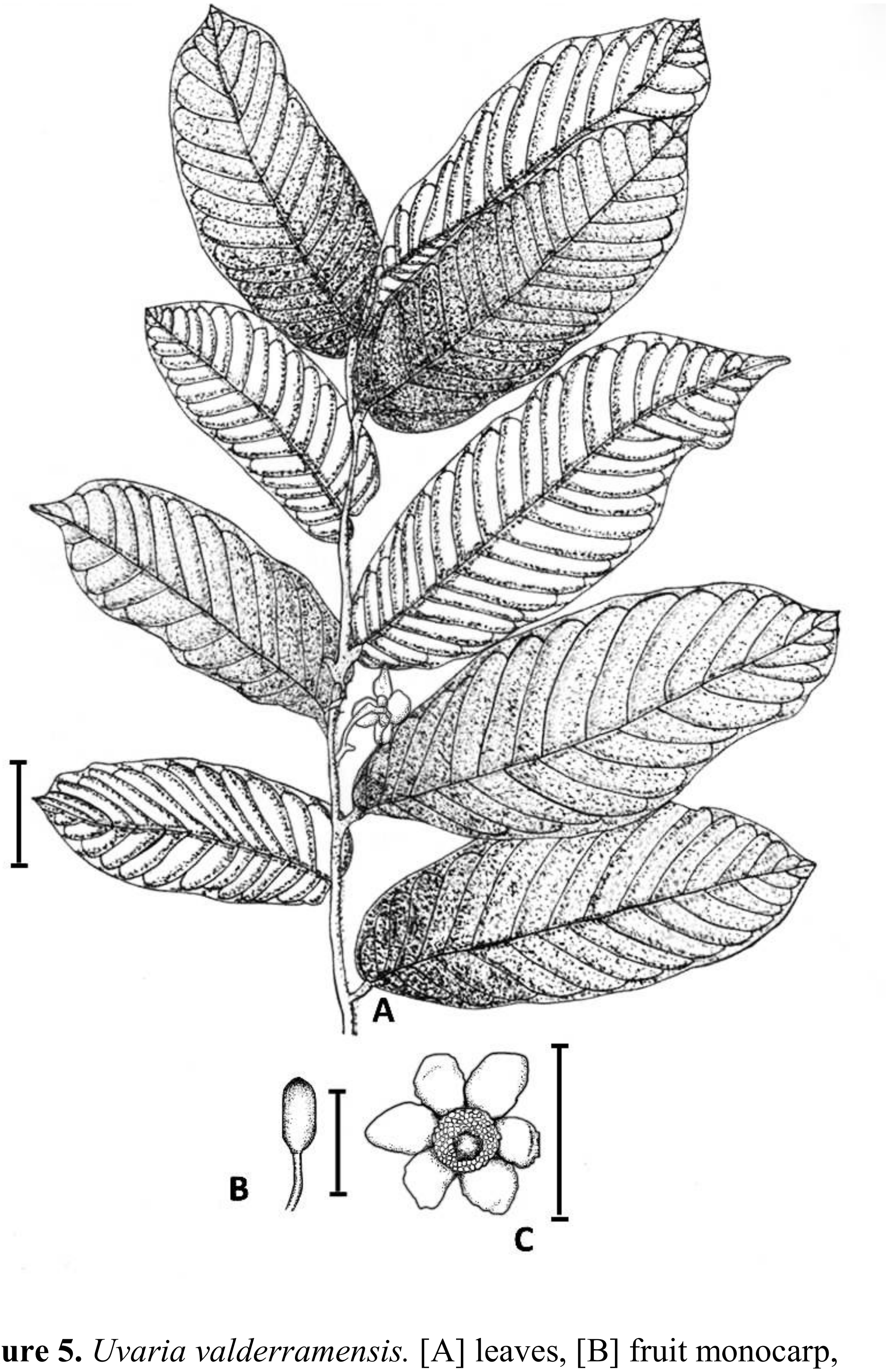

Figure 5. *Uvaria valderramensis.* [A] leaves, [B] fruit monocarp, [C] flower bud, [D] flower. (Scale bars: A=8 mm, B=17 mm and C=28 mm)

Table 1. Sequence characteristics with tree statistics of the separate and combined datasets.

	***trn*L-F**	***mat*K**	**combined sets**
Number of taxa	70	70	70
Matrix length	1094	849	1943
Number of parsimony informative characters	164	182	346
Number of most parsimonious trees	3050	1831	4881
Tree length	320	342	662
Consistency index	0.688	0.683	0.678
Retention index	0.864	0.862	0.859

Table 2. Positive results for metabolites in each type of extract

Petroleum ether layer extract	**DCM layer extract**	**Butanol extract**
Triterpenes	Phenols	Alkaloids
Sterols	Tannins	
	Flavonoids	